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Development of a reversed-phase high-performance liquid chromatography analytical methodology for the determination of antihypertensive peptides in maize crops

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ABSTRACT

The aim of this work was to estimate the content of three highly antihypertensive peptides (LQP, LSP, and LRP) in different maize crops. For that purpose, a method consisting of the extraction of the protein containing these peptides (α-zeins), releasing of peptides by thermolysin digestion, and separation and detection of peptides was designed. The rapid and efficient ultrasound assisted extraction of α-zeins proteins from whole maize kernels was achieved using 70% of ethanol followed by precipitation with acetone. A 10 mM Tris–HCl (pH 8.0) buffer containing 8 M urea enabled to dissolve the precipitated α-zeins. This buffer was diluted to reach a 6 M urea concentration before digestion to keep active the enzyme. Other digestion parameters that were optimized were: enzyme to substrate ratio (5:100 was selected), digestion temperature (50 °C) and digestion time (6 h). The RP-HPLC separation in a fused-core column was also optimized allowing the separation of the three peptides extracted from maize kernels in 6 min. The presence of the three antihypertensive peptides in the digested extract was confirmed using HPLC–Q-TOF-MS analysis and by comparison with peptide standards. Clear differences were observed in the content of the three antihypertensive peptides and, thus, in the antihypertensive activity of the analyzed crops. The content of LRP peptide was very low regardless of the maize variety while the content of LQP and LSP significantly varied among studied maize lines.

1. Introduction

Hypertension appears as a leading cause of cardiovascular diseases (CVDs) and is known as 'silent killer' since over 50% of hypertensive population is unaware of their condition [1]. According to the World Health Organization, hypertension is a highly prevalent cardiovascular risk factor worldwide and its treatment has been shown to prevent CVDs [2]. Different therapies can be applied to prevent hypertension being the use of angiotensin converting enzyme inhibitors (ACE inhibitors (e.g. captopril)) the first choice [3]. ACE inhibitor compounds act on the renin–angiotensin system associated in the control of blood pressure in living organisms. Angiotensin I is hydrolyzed in the presence of angiotensin I converting enzyme (ACE) to angiotensin II, leading to an increase in blood pressure. The ACE also removes a dipeptide from the C-terminus of bradykinin resulting in the inactivation of this vasodilator. As a consequence, ACE inhibitors cause effective reduction of blood pressure by decreasing the angiotensin II level and rising up bradykinin level [4–6]. Most antihypertensive drugs employ mechanism of ACE inhibition. Synthetic drugs are very potent but they also provoke several adverse effects [5,7]. An alternative can rise from those foods naturally containing antihypertensive peptides which do not yield adverse effects [8,9].

Most ACE inhibitory peptides contain 2–12 amino acids residues with a noticeably amount of hydrophobic amino acids such as proline, especially at C-terminal position [10]. ACE inhibitors were found in marine foods [11], fishes [8], meat [12], vegetable foods [13], mushrooms [14], and processed products [15]. Most studied antihypertensive peptides are from foods of animal origin, specially dairy products [16–18], although the most active peptides were found in maize. Indeed, maize contains three peptides (Leucine-Glutamine-Proline (LQP), Leucine-Serine-Proline (LSP), and Leucine-Arginine-Proline (LRP)) derived from the α-zein protein fraction with extremely high antihypertensive activity (IC_{50} value (the half maximal inhibitory concentration) 2.0, 1.7, 0.29 μM, respectively) [19] which is much higher than the popular Valine-Proline-Proline (VPP) (IC_{50} = 9.13 μM) and Isoleucine-Proline-Proline (IPP) (IC_{50} = 5.15 μM) found in milk. Taking into account that the protein content of maize crops can vary [20] and that antihypertensive activity of peptides is highly dosage dependent [21,22], the development of analytical methodologies for estimating peptide contents in different crops is required.

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Worldwide corn or maize (*Zea mays* L.) is a major crop for both livestock feeding and human nutrition [23]. Maize protein content is in the range 6–12% (as dry basis) [20]. Zeins, according to Osborne nomenclature, are the prolamin fraction of maize, representing 60% of total maize proteins [24]. Zeins can be classified as: α-zeins (21–25 kDa), β-zeins (17 kDa), γ-zeins (18 and 27 kDa), and δ-zeins (10 kDa). The most abundant zein is the α fraction accounting for 75–85% of total zeins [25]. Two major groups of α-zeins can be separated using SDS-PAGE: Z19 zein migrating at 19 kDa and Z22 zein 22 kDa. Nevertheless, the studies of zein sequences obtained from cloned cDNAs and genes, have shown that those two groups of zeins had a Mw around 23–24 and 26–27 kDa, respectively [26].

Several different attempts were made in order to obtain total-zeins from maize kernels, where extraction using aqueous solutions of ethanol or isopropanol with or without a reducing agent are the most frequent [27–29]. Moreover, despite there is one methodology enabling α-zeins extraction, it was applied to maize product with high protein content (corn gluten meal, CGM) [19]. Nevertheless, to our best knowledge, none of these procedures have been applied to exclusively extract the α-zeins from maize kernels.

Next step in the isolation of antihypertensive peptides would be the digestion of α-zeins. Different enzymes have been employed for the digestion of proteins containing antihypertensive peptides being thermolysin the most preferred due to its broad specificity to hydrophobic amino acids [30]. Reports about digestion of CGM by trypsin [31], alcalase [32], thermolysin [19], and six different commercial proteases [33] or zeins by trypsin or thermolysin [34,35] can be found in the literature. Nevertheless, in all cases CGM or zeins were purchased, and no extraction procedure was previously applied. The lack of methodologies where zein proteins were digested after extraction from whole maize kernels need to be highlighted since the selection of a suitable buffer enabling to dissolve the alcohol soluble α-zeins and to keep active the enzyme had to be overcome. Moreover, comparison of digestion protocols using thermolysin revealed that digestion conditions differed significantly from one work to the other.

Regarding peptide separation, a new trend in HPLC is focused to the development of stationary phases enabling high sample throughput analysis of peptides. Several strategies have been developed being the use of fused-core or superficially porous silica particles very interesting for the reduction of analysis times while keeping column efficiency and low back pressure. Columns with 2.7 μm fused-core particles produce approximately half of the back pressure of the 1.8 μm conventional columns allowing the use of traditional HPLC systems [36]. This fused-core particles start to play important role in chromatography and their use in bio-analytical methods have already been reviewed [37]. Nevertheless, the use of this innovative approach is still not common, and has scarcely been employed for peptide separation.

The aim of this work was to develop an analytical methodology for the rapid extraction of α-zeins from maize kernels allowing their further digestion by thermolysin and their separation by RP-HPLC in order to evaluate the content of three highly active antihypertensive peptides (LQP, LRP, and LSP) in maize crops.

2. Materials and methods

2.1. Chemicals and samples

Water, was freshly taken every day from a Milli-Q system (Millipore, Bedfore, MA, USA). All used reagents were of analytical grade purity. Acetic acid (AA), AA with purity for LC–MS, acetone, acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), isopropanol (IPA), and urea were supplied from Scharlau Chemie (Barcelona, Spain). Formic acid (FA), hydrochloric acid, sodium dodecyl sulfate (SDS), sodium hydroxide, tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), and β-mercaptoethanol (B-ME) were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide, dithiothreitol (DTT), iodoacetamide (IAM), thermolysin, and trifluoroacetic acid (TFA) were from Sigma (St. Luis, MO, USA). Heptafluorobutyric acid (HFBA), and sodium acetate were acquired from Fluka (Burchs, Switzerland) and trichloroacetic acid (TCA) was from Panreac (Barcelona, Spain). All chemicals and gels for SDS-PAGE analysis were acquired in Bio-Rad (Hercules, CA, USA): Laemmli buffer (62.5 mM Tris–HCl, 25% (v:v) glycerol, 2% (m:v) SDS, 0.001% (m:v) bromophenol blue), Mini-Protean Precast Gels, running buffer (25 mM Tris–HCl, 192 mM glycine, and 0.1% SDS, pH 8.3), Precision Plus Protein Standards, and Bio-Safe Coomassie stain. Standards and samples employed were: corn gluten meal (CGM) (Sigma, St. Luis, MO, USA), peptides LQP, LSP, and LRP (GeneScript Corp., Piscataway, NJ, USA), standard of zeins (Sigma, St. Luis, MO, USA), and maize lines that were kindly donated by a Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain): EZ6, B73, EZ11A, EZ9, A632.

Prepared solutions were stored in the fridge at 4 °C with the exception of urea, IAM, and DTT solutions that were always freshly prepared. Additionally, thermolysin powder or thermolysin stock solution (2.5 mg/mL in water) and peptides were stored always at −20 °C. Standards of peptides (1 mg/mL or 0.1 mg/mL) were dissolved in water (LRP, LQP) or in ACN (LSP) according to the recommendation guide supplied by Genscript. Standard of zeins (100 mg/mL) was dissolved in 70% of ethanol prior to analysis. Maize kernels (around thirty whole kernels for each line) were grounded with a domestic miller during 1 min at ambient temperature. All milled maize powders were stored at 4 °C.

2.2. Extraction and fractionation of zeins from CGM

Main maize proteins (zeins) were fractionated following a method developed by Parris and Dickey [38] and improved by Rodríguez-Nogales et al. [39] with some modifications. The method was applied to fractionate zeins from CGM. The method consisted of extracting 2 g of CGM with 20 mL of 60% IPA containing 1% B-ME at room temperature and centrifuging for 1 min at 4000 × *g*. This extraction was repeated three additional times. Next, three volumes of 100% IPA were added and the resulting solution was left overnight at 4 °C. Afterwards, the solution was centrifuged for 10 min at 4000 × *g* and 4 °C. The resulting pellet containing β- and γ-zeins was separated from the supernatant. The supernatant was mixed with two volumes of water and 0.01 volumes of sodium acetate (pH 6.0). The solution was left for 2 h followed by centrifugation for 10 min at 4000 × *g* and 4 °C. The pellet containing α-zeins was separated.

2.3. Extraction and purification of α-zeins from maize lines

α-zeins from the EZ6 maize line were extracted using a method developed by Yano et al. [19] to isolate α-zeins from CGM. The method was carried out by extracting 1 g of pulverized maize line with 10 mL of 70% EtOH. For a more efficient and fast extraction, an ultrasonic probe (VCX.130, Sonic Vibra-Cell, Hartord, CT, USA) was employed for 10 min (amplitude 90%) followed by centrifugation (4000 × *g*, 10 min, 20 °C). α-Zeins in the previous extract were purified by precipitation with 80 mL of acetone containing 0.07% B-ME followed by centrifugation (4000 × *g*, 15 min, 4 °C). The resulting

pellet was dissolved in 20 mL of a buffer (10 mM Tris–HCl (pH 8.0) containing 8 M urea) and left overnight at 4 °C.

2.4. Ultrafiltration

The protein extract obtained with 70% EtOH was ultrafiltrated through semi permeable membranes with Mwco of 3 kDa (Amicon® Ultra, Millipore) and 10 kDa (Centricon®, Millipore), for 20 min by centrifugation at room temperature (4000 × *g*).

2.5. α-Zein digestion

Purified α-zeins dissolved in a buffer containing 8 M urea were diluted in water to obtain a final urea concentration of 6 M. Optimized digestion protocol consisted of mixing 1.5 mL of protein extract with thermolysin stock solution (2.5 mg/mL), so that the enzyme to substrate ratio was 5:100 (w:w). Solution was incubated in a hot air oven (Memmert, model 300, Schwabach, Germany) for 6 h at 50 °C, then boiled for 10 min, and centrifuged for 5 s (mini centrifuge Nahita, model 2507, 7200 rpm).

2.6. HPLC analysis

Separation of zein proteins was performed on a modular Agilent Technologies liquid chromatograph (Pittsburg, PA, USA). The chromatographic assembly consisted of a degassing system, a quaternary pump, a thermostatized compartment for the column, an injection system, and an UV detector (series 1100). HP Chemstation software was used to control HPLC instrument. All experiments were made by duplicate and injected twice into the HPLC system.

Extracted and purified zeins were separated in a POROS R2/10 perfusion column (100 mm × 2.1 mm I.D.) (Perspective Biosystem, Framingham, MA, USA) using a flow-rate of 1 mL/min, a temperature of 25 °C, a binary gradient from 5–50.2% B in 7.2 min, 50.2–65.4% B in 2.94 min, 65.4–95% B in 1 min, and 95–5% B in 1 min. Mobile phases consisted of Milli-Q water/0.1% (v:v) TFA (mobile phase A) and ACN/0.1% (v:v) TFA (mobile phase B). The injected volume was 5 μL and the detection was performed at a wavelength of 280 nm.

Separation of digested α-zein and target peptides was performed by HPLC with UV detection using an Ascentis Express Peptide ES-C18 column (100 mm × 2.1 mm I.D., with 2.7 μm particle size) with an Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 μm particle size) both from Supelco (Bellefonte, PA, USA). The optimized chromatographic conditions for the separation of peptides were: 3% B for 5 min, 3–5% B in 5 min, 5–97% B in 2 min, and 97–3% B in 2 min; mobile phase A, Milli-Q water/20 mM AA; mobile phase B, ACN/20 mM AA; temperature, 40 °C; flow-rate, 0.4 mL/min; injected volume, 5 μL; UV detection at 210 nm. Digested extracts were filtrated through 0.45 μm pore size regenerated cellulose filter membranes (Titan 2, Eatontown, NJ, USA) before injections.

2.7. MS analysis

MS analysis was performed using a Quadrupole-Time-of-Flight (Q-TOF) MS (instrument series 6530) from Agilent Technologies coupled to a liquid chromatograph 1100 series also from Agilent Technologies. HPLC separation was made on the Ascentis Express column previously employed, using mobile phases: A, Milli-Q water/0.3% (v:v) AA and B, ACN/0.3% (v:v) AA. The elution gradient was 5–20% B in 15 min, 20–95% B in 2 min, and 95–5% B in 2 min with a flow-rate of 0.5 mL/min and a column temperature of 25 °C. The injected volume was 20 μL for the digested extract and 1 μL for the standards of peptides. Simultaneous UV (210 nm) and MS detection were registered. The mass spectrometer was operated with the ESI source Jet Stream in the positive ion mode using only TOF analyzer (only MS mode) and a mass range of 100–3200 *m*/*z*. The dry gas flow-rate was 10 L/min and its temperature was 300 °C. The nebulizer gas pressure was 30 psig and the sheath gas flow and temperature were set up at 12 L/min and 400 °C, respectively. MS conditions were: capillary voltage, 3500 V; fragmentator, 200 V; skimmer voltage, 60 V; octopole voltage, 750 V, and nozzle voltage, 0 V. Mass spectrometer control, data acquisition, and data analysis were carried out with the MassHunter Software.

2.8. SDS-PAGE analysis

A Bio-Rad Mini-Protean system was used for the electrophoretic separation of proteins. Samples were prepared as follows: 15 μL of each sample was added to 15 μL of Laemmli buffer containing 0.5% of B-ME, vortexed, boiled at 95 °C for 5 min, and centrifuged for 10 s. Separation was carried out on commercial Mini-Protean Precast Gels using a solution consisting of 25 mM Tris–HCl, 192 mM glycine, and 0.1% SDS (pH 8.3) as running buffer. Electrophoresis was performed by applying 200 V for 30 min. Protein standards (Precision Plus Protein Standards) consisting of recombinant proteins expressed by *Escherichia coli* (with Mw 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa) were injected in the first lane and used as a ladder to estimate molecular weights of proteins in the following lanes. After electrophoresis, proteins were fixed by gentle agitation in 100 mL solution consisting of 10% (v:v) glacial acetic acid and 40% (v:v) MeOH for 30 min, stained for 1 h (gently agitating) with 50 mL of Bio-Safe Coomassie stain, and washed with Milli-Q water for at least 2 h.

3. Results and discussion

3.1. Selection of the extraction procedure and identification of α-zeins

There is no quick and efficient procedure to extract α-zeins from maize kernels. Existing extraction procedures are mainly focused to the extraction of whole maize proteins or total-zeins from maize kernels or to the extraction of α-zeins from maize products with high protein content, e.g. CGM.

The procedure developed by Parris and Dickey [38] and improved by Rodríguez-Nogales et al. [39] enabled the fractionation of zeins from CGM based on their different solubility. We have modified the original fractionation procedure reducing the second overnight precipitation to 2 h. Moreover, we have also tried the use of this method to fractionate zeins from maize kernels. Nevertheless, zein fractionation from maize kernels was not possible due to their low protein content (6–12%) in comparison with maize products (CGM contained 60% protein) employed in the original procedure. Then, the method was applied to the extraction of zeins from CGM such as it was previously performed by Parris and Dickey [38]. The precipitated α, and β/γ-zeins, and the supernatant obtained from CGM, were injected into the HPLC system. Although, in each fraction different peaks were distinguished, small signals supposedly from α-zeins, could also be observed in the β/γ-zein fraction, and in the supernatant. These results could indicate that part of the analytes remain in the solution after precipitation. These results suggested that this fractionation method was not quantitative and could not fully separate α-zeins from β and γ-zeins. In order to obtain a clear identification of peaks corresponding to α-zeins, an extraction procedure used for extracting α-zeins from CGM [19] was applied to maize kernels from the EZ6 line. The extraction consisted of mixing 1 g of maize kernel with 10 mL of 70% EtOH. The extraction procedure was accelerated by the employ of an ultrasonic probe (20 min, 100% amplitude). Finally,

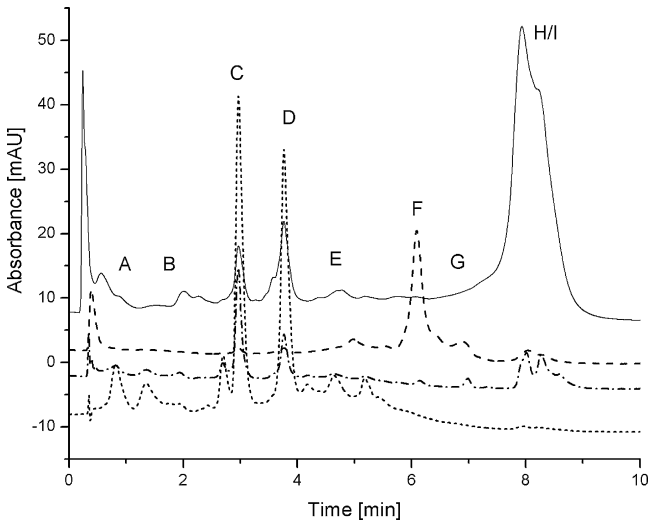

Fig. 1. Chromatograms obtained for: the commercial α-zein standard (solid line); fraction β/γ-zeins obtained by the fractionation of CGM (dash line); the extract of α-zeins obtained from the maize kernel with 70% of EtOH (dash dot line); extract when passing through a membrane with Mwco of 3 kDa (dot line). Chromatographic conditions: column: POROS R2/10 perfusion (100 mm × 2.1 mm I.D.), mobile phases: A, Milli-Q water/0.1% (v:v) TFA; B, ACN/0.1% (v:v) TFA; gradient: 5–50.2% B in 7.2 min, 50.2–65.4% B in 2.94 min, 65.4–95% B in 1 min, 95–5% B in 1 min; T = 25 °C; inj. vol. = 5 μL; F = 1 mL/min; λ = 280 nm.

the extract was centrifuged for 10 min (4000 × *g*, 20 °C). Furthermore, the 70% EtOH extract, supposedly containing α-zeins, was subjected to ultrafiltration through membranes with Mwco 3 and 10 kDa, where in both cases, results were identical. On the other hand, a commercially available zeins standard mainly made up from α-zeins (due to the fact that other types of zeins (β, γ, δ) are thought to contribute to gelling [40]) was also used in order to clarify the identification of α-zeins. The commercially available zeins standard, the precipitated β/γ-zeins, the extract of α-zeins obtained from maize kernels extracted with 70% of EtOH, and the same extract passing through the membrane with Mwco 3 kDa were injected into the HPLC system (Fig. 1). α-Zeins eluted in three peaks at 3, 3.7, and 8 min (peaks C, D, and H/I). As previously stated, β and γ-zeins fraction also yielded small peaks C, D, and H/I in addition to a big signal at 6 min (peak F) and two small signals (peaks E/G). Extract obtained from maize kernels with 70% of EtOH using the method of Yano et al. [19] accelerated with an ultrasonic probe, yielded mainly peaks C, D and H/I in addition to small signals A, B, and E. Interestingly, when this extract was subjected to ultrafiltration through a 3 kDa Mwco membrane, peaks H/I disappeared and only signals A–E were observed. From these results, α-zeins seemed to elute mainly in peaks H and I, while peaks F and G could correspond to β- and γ-zeins, and peaks A–E (passing through the 3 kDa filter) could be small molecules. These small molecules could correspond to impurities like polyamines putrescine compounds which have very high UV absorbance at 320 nm [27]. As reported Moreau et al. [41] when maize kernels are extracted with common polar organic extractants (e.g. ethanol or methylene chloride), high levels of polyamine conjugates such as diferuloylputrescine (DFP) and *p*-coumaroylferuloyputrescine (CFP) were also extracted. Moreover, LC–MS analysis of these compounds reported molecular weights of 440 and 410 mass units, for DFP and CFP, respectively [42] demonstrating these compounds passed through the 3 kDa cut-off filter. Furthermore, the chromatogram obtained when registering the signal at 320 nm (specific wavelength for polyamine putrescine compounds) showed just peaks A, B, C, D, and E and not the peaks assigned to zeins (F, G, and H/I). Moreover, the extract obtained from maize kernel using 70% of ethanol and accelerated with ultrasonic probe was also analyzed by SDS-PAGE. The electrophoretic pattern of this extract confirmed the presence of only α-zeins bands appearing at molecular weights of 19 and 22 kDa.

3.2. Optimization of α-zeins extraction

Once demonstrated that the use of an organic solvent as ethanol could extract α-zeins from maize kernels, following parameters were next optimized: organic solvent, percentage of organic solvent in extractant, time of extraction, amplitude of the ultrasonic probe, and maize to extractant ratio. According to the literature, an alternative solution to EtOH for the extraction of α-zeins could be IPA and ACN [28]. ACN was discharged due to the low amount of α-zeins that were extracted and due to the high amount of interferences that were obtained. Regarding IPA and EtOH, different solutions ranging from 40% to 90% were tried for the extraction of α-zeins. Maximum α-zein extraction with EtOH was reached at 70% while for IPA, the highest extraction was obtained at 60%. Taking into account that IPA extraction provided higher amount of interferences and the higher price of this solvent, 70% EtOH was chosen as the optimum extractant for α-zeins. Next, the influence of the extracting time using the ultrasonic probe was evaluated in the range from 0 (sample mixed with solvent and centrifuged) to 60 min in 10 min intervals. Results indicated that α-zein solubility increased up to 10 min and afterwards decreased, concluding the optimum time for extraction was 10 min. Next, the ultrasonic energy was modified to evaluate its effect on the amount of extracted proteins. Following amplitudes were tested: 20%, 30%, 50%, 70%, 90%, and 100%. Results showed that higher ultrasonic energies accelerated the extraction of α-zeins up to 90% of amplitude, that was selected as the optimum value. Moreover, different sample to solvent ratios were evaluated: 1:3, 1:5, 1:10, 1:15, and 1:20 (w:v). Extractions under sample to solvent ratios 1:5 and 1:3 were not possible and gave unreliable results. A 1:10 (w:v) sample to solvent ratio was further used as optimum since it enabled to obtain the maximum peak area. After optimization of the extraction procedure, a study on the stability of samples was performed. The same sample was injected over a long period of time (26 h). No change in the peak area corresponding to α-zeins was observed. Estimated repeatability for the ten first injections of the same sample yielded a RSD of 0.4%. The precision obtained when injecting eight different samples in the same day was 2.2%. The inter-day precision when injecting eight maize samples in two different days (16 analysis) was 3.4%.

3.3. Purification of α-zeins

In order to clean the α-zeins extract from interferences and also to transfer α-zeins to other suitable solution for enzymatic digestion, the precipitation of proteins was proposed. Two different precipitation methods were designed. Precipitation methods consisted of mixing α-zeins extract with 50 mL of 10% TCA/acetone containing 0.07% B-ME or with 50 mL of acetone containing 0.07% B-ME, both at −20 °C for 1 h. Both pellets were dissolved again in 70% EtOH and were injected into the chromatographic system. Pellets obtained by the TCA/acetone method were very difficult to redissolve. Unlike this procedure, pellets from the acetone methodology were quickly redissolved in 70% EtOH. Moreover, a recovery close to 100% was observed by comparing the signal corresponding to the protein before its precipitation with that of the protein after its precipitation and redissolution. In order to use the best conditions for the acetone precipitation of α-zeins, the volume of acetone and the precipitation time were optimized. The recoveries of α-zeins and interferences were estimated taking into account signals obtained before and after precipitation with different volumes (50–100 mL) of acetone. Results data showed that all samples, regardless to the

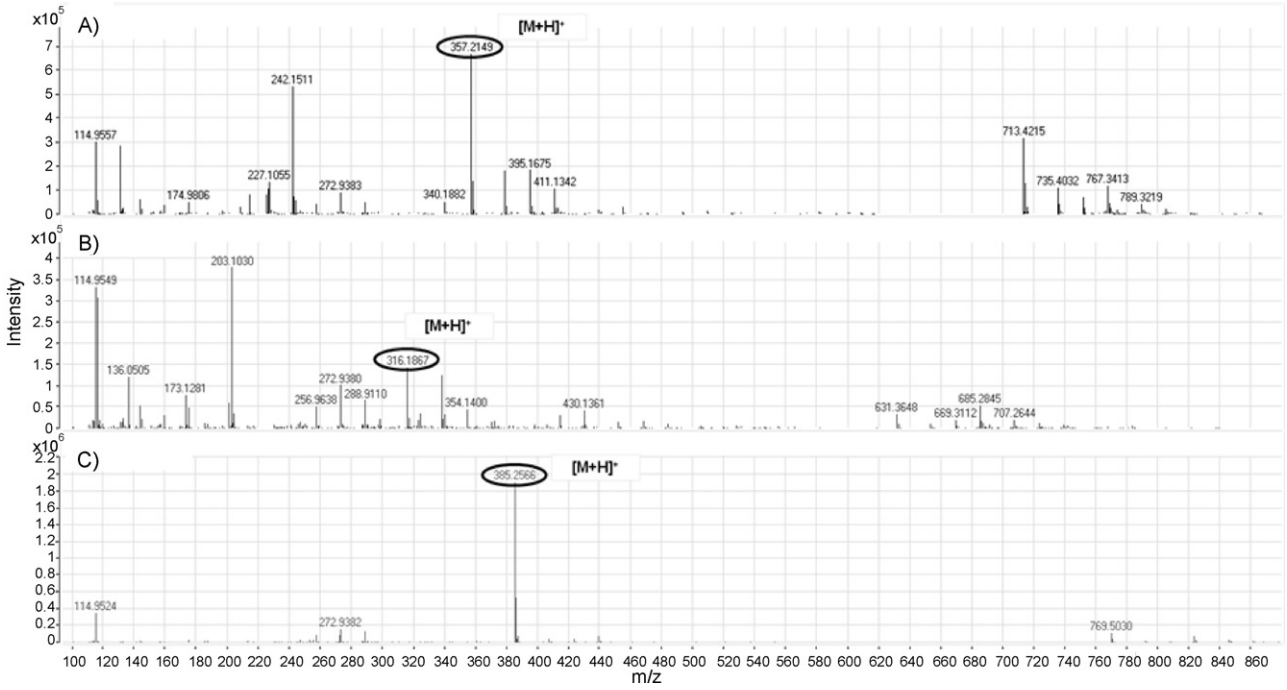

Fig. 2. MS spectra of standard peptides: (A) LQP, (B) LSP, and (C) LRP. Identified ions from each peptide are marked by circle.

volume of acetone employed, were cleaned from interferences at approximately the same level. Finally, an acetone volume of 80 mL was considered as the optimum one since it yielded the highest protein recovery. Moreover, different precipitation times at −20 °C were tested (0–90 min in 30 min intervals). Results at each precipitation time were very similar and no waiting time for precipitation was considered necessary.

3.4. Solubilization of precipitated α-zeins in an aqueous buffer

The selection of an appropriate solvent for the enzymatic digestion of a protein is usually not a big problem since most proteins are soluble in aqueous buffers where enzymes are working. Nevertheless, since zeins are alcohol soluble proteins, choosing a medium in which zeins were soluble and, simultaneously, enzyme was active, is quite challenging. Information found in the literature indicated that zeins were soluble in aqueous solutions containing high concentrations of urea, salts, and ammonia [20]. Moreover, thermolysin has unusual properties and remains active in unfolding conditions such as high concentrations of urea (6–8 M urea) [43] or in the presence of some salts [44]. In addition, anionic surfactants like SDS at concentrations up to 1% had been proven to be useful for solubilization of proteins [41]. Taking into account this bibliographic information, the following media were employed for the solubilization of the α-zeins obtained from the maize kernels: 4 M urea/0.1% NH_3; 10 mM Tris–HCl/6 M urea (pH 8.5); 10 mM Tris–HCl/8 M urea (pH 8.5); 50 mM Tris–HCl/5 mM $CaCl_2$ (pH 8.0); 8 M urea; 1% NH_3; 10 mM Tris–HCl/0.1% SDS (pH 8.5); 10 mM Tris–HCl/8 M urea + 0.6% SDS (pH 8.5); 10 mM Tris–HCl/0.6% SDS (pH 8.5). Nevertheless, only the buffer consisting of 10 mM Tris–HCl/8 M urea (pH 8.5) could dissolve α-zeins and enabled the digestion with thermolysin by the previous dilution of urea to 6 M. This solubilization process was accelerated when temperature decreased. Hence, the precipitated α-zeins were best dissolved when keeping in the fridge overnight. Obtained digested extract was injected into the HPLC system using UV detection at 210 nm which is a suitable wavelength for detecting peptides. Comparison of chromatograms demonstrated (data not shown) that α-zeins peaks appearing in the initial extract disappeared after thermolysin digestion and, at the same time, new signals corresponding to peptides appeared. Nevertheless, the perfusion column did not permit a good separation of peptides and it was replaced by a fused-core column in next experiments.

3.5. Identification of target peptides in the α-zeins digested extract

In order to confirm that target peptides LQP, LRP, and LSP were in the digested extract, HPLC–MS was used. For that purpose, the chromatographic conditions were chosen in order to obtain the best peptide separation with the fused-core column and, at the same time, a nice environment for the MS detection. At this point, it is important to highlight that the ion-pairing reagent used in this work up to now in RP-HPLC separations was not suitable for the MS detection. In fact, TFA creates strong complexes with peptides which are enhancing the separation in the RP-HPLC column, but at the same time, these complexes are strong enough to inhibit peptide ionization in MS resulting in signal suppression. Different alternatives such as reduction of the TFA concentration or the use of other ion-pairing reagent can be proposed. Typical mobile phases used for HPLC with MS detection are 0.3% AA, 0.2% HFBA, 0.025% TFA or 0.2% FA [45]. These ion-pairing reagents were tested for the separation of the α-zein digested extract. The chromatogram obtained using AA as ion-pairing reagent seemed to enable the best separation of peptides. As a consequence, 0.3% of AA in the mobile phase was chosen for further experiments using the following gradient: 5–20% B in 15 min, 20–95% B in 2 min, and 95–5% B in 2 min. The comparison of the elution times of these peaks with the elution times of the standard peptides enabled a tentative identification of the peaks corresponding to LQP, LSP and LRP. Moreover, the MS spectra of these peptides in the maize sample and in the standards were compared for a better identification. Fig. 2 shows the spectra corresponding to the three standard peptides. All three peptides in the standards resulted singly protonated. In the case of LQP and LRP, this protonated ion was the base peak. Moreover, additional signals were also observed in every peptide standard, especially in

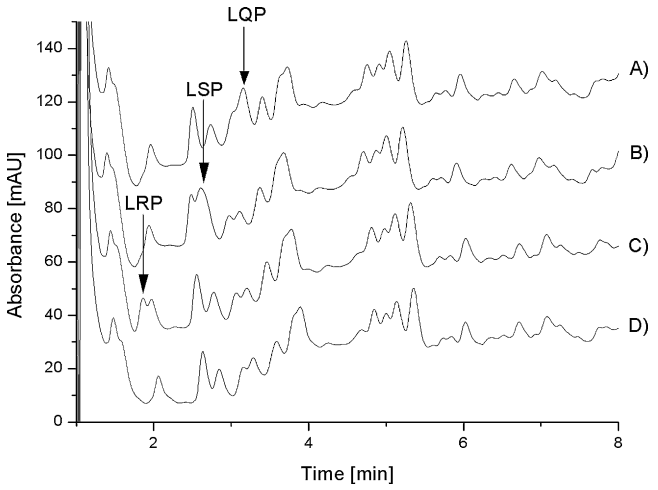

Fig. 3. Chromatograms corresponding to the digested extract spiked with (A) LQP, (B) LSP, (C) LRP and to (D) the digested extract. Chromatographic conditions: column: Ascentis Express Peptide ES-C18 (100 mm × 2.1 mm I.D., with 2.7 μm particle size) with Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 μm particle size), mobile phases: A, Milli-Q water/0.3% AA (v:v), B, ACN/0.3% AA (v:v). Gradient: 5–20% B in 15 min, 20–95% B in 2 min, 95–5% B in 2 min, T = 25 °C, inj. vol. = 10 μL, F = 0.5 mL/min, λ = 210 nm.

the case of LQP and LSP. These additional signals could correspond to fragments of peptide and other reagents that have resulted during their synthesis. The total ion chromatogram (TIC) of the digested extract, and the extracted ion chromatograms (EICs) of each peptide ion obtained from the separation of digested extract, and peptide standards were compared in order to identify target peptides in the digestion extract. Moreover, the digested extract of α-zeins was spiked with every peptide standard to confirm the identity of peaks corresponding to these peptides in the digested extract. The digested extract (250 μL) was enriched with 2 μL of LQP (1 mg/mL), LSP (0.1 mg/mL) or LRP (1 mg/mL) (Fig. 3). Comparison of spiked and non-spiked extracts enabled the clear identification of LQP, LSP, and LRP peptides.

3.6. Optimization of the chromatographic separation of the α-zeins digested extract by RP-HPLC

Once target peptides have been identified in the digested extract, the chromatographic conditions were optimized. Since target peptides still eluted closely in the first minutes of the chromatogram, the slope of the gradient in the first part of the separation was reduced. Finally, a gradient consisting of a first isocratic step at 3% B for 5 min and followed by 3–5% B in 5 min, 5% B in 5 min and 5–97% B in 2 min was chosen for further experiments. Different concentrations of AA in the mobile phase (20, 40, 50 (0.3%), 60, and 80 mM) were also examined. Separation was not possible when removing the AA reagent, while high concentrations resulted in very low retention. Therefore, a concentration of 20 mM of AA was found to be the best. Moreover, four different temperatures were tried: 25 °C, 30 °C, 35 °C, and 40 °C. Best separation was obtained at 40 °C. Nevertheless, despite the appropriate separation of standard peptides (LRP peptide eluted at 2.1 min, LSP at 2.7 min, and LQP at 3.9 min), the digested extract from the maize kernel showed a limited sensitivity and very small peaks were observed. Thus, next step was focused to increase the sensitivity.

3.7. Optimization of thermolysin digestion of α-zeins

The following parameters were optimized: concentration of Tris–HCl buffer, pH, substrate to enzyme ratio, temperature, and digestion time. Also, an attempt of enhancing the digestion using an ultrasonic probe and the influence of reduction and alkylation of proteins previous to digestion, have been evaluated. Digestion can be enhanced and accelerated if using ultrasonic energy. The digested extract prepared under the initial conditions (α-zeins dissolved in a 10 mM Tris–HCl/8 M urea buffer (pH 8.5) and diluted to 6 M urea was mixed with thermolysin at an enzyme-to-substrate ratio of 0.1:100 (w:w) and digested at 37 °C for 3 h) was subjected to the action of the ultrasonic probe. Both the amplitude (20%, 40%, 60%, 80%) and the time (3, 5, 7, 10 min) were studied. Surprisingly, in all cases proteins remained undigested. These results suggested that thermolysin was not able to stand the ultrasonic energy resulting inactive under these conditions. Therefore, precipitated zeins were dissolved in 20 mL of a buffer containing 8 M urea and different concentrations (10, 20, 50 or 100 mM) of Tris–HCl (pH 8.5). Best results were obtained when using the 10 mM Tris–HCl buffer. On the other hand, according to the literature, the stability pH range of thermolysin ranges from 5.0 to 9.5 [46]. In order to confirm the pH which was optimum for the digestion of α-zeins, the following buffer pHs were evaluated: 7.5, 8.0, 8.5, and 9.5. Results showed that thermolysin worked best at pH 8.0, while at pH 9.0 the digestion was inhibited due to instability of the enzyme. Following, the ratio enzyme to substrate was optimized. Several enzyme to substrate ratios were tested: 0.02:100, 0.1:100, 0.5:100, 1:100, 2.5:100, 5.0:100, and 10:100 (w/w). Moreover, blank digestions without α-zeins were also performed for every ratio in order to evaluate the existence of autodigestion [47]. Rising up the level of enzyme resulted in an increase of detected peptides till ratio 5:100. No autodigestion was observed at enzyme to substrate values below 5:100 while at a ratio 10:100, peaks probably corresponding to the hydrolyzed enzyme were observed. As a consequence, a 5:100 ratio was chosen. Furthermore, the influence of temperature in the digestion was next studied. Since themolysin can stand temperatures up to 70 °C, examined temperatures were: room temperature, 37 °C, 50 °C, 60 °C, and 70 °C. Maximum signals were observed at 50 °C that was chosen as optimum temperature value. Different digestion times were also tested (1, 3, 6, 12, 18, 24 h). Despite there were not significant changes in the profiles obtained, results showed a slight improvement in the hydrolysis of α-zeins till 6 h. Therefore, 6 h of digestion was chosen as optimum time. Moreover, we evaluated the effect of reduction and alkylation of α-zeins previously to their digestion. Interestingly, the hydrolysis of α-zeins did not occur when reduction and alkylation took place. Finally, the injected volume was optimized. Volumes ranging from 2 to 20 μL were employed. Reduction of the injected volume decreased the height of the detected signals. However, signals were much better resolved at reduced injected volumes and an injection volume of 5 μL was finally adopted. Fig. 4 shows the separation of the digested extract under the final chromatographic and digesting conditions. The arrows show the peaks in which target peptides are eluting.

3.8. Application of the developed methodology to the analysis of maize varieties

The developed methodology for the extraction and purification of α-zeins was applied to different maize varieties to evaluate the performance of the optimized methodology for isolating α-zeins. The worst purification was obtained for the crop B73 and the best for the EZ9 crop. However, the recovery of proteins after their precipitation with acetone was always higher than 93%. Peptidic profiles were qualitatively very similar and only differences on the size of the peaks corresponding to the target peptides were observed. The comparison of the average areas of every antihypertensive peptide in the studied maize crops is shown in Fig. 5. There were statistically significant differences among results obtained for every antihypertensive peptide in the studied maize crops (ANOVA, $P < 0.05$). The content in LRP peptide was very low in all the analyzed

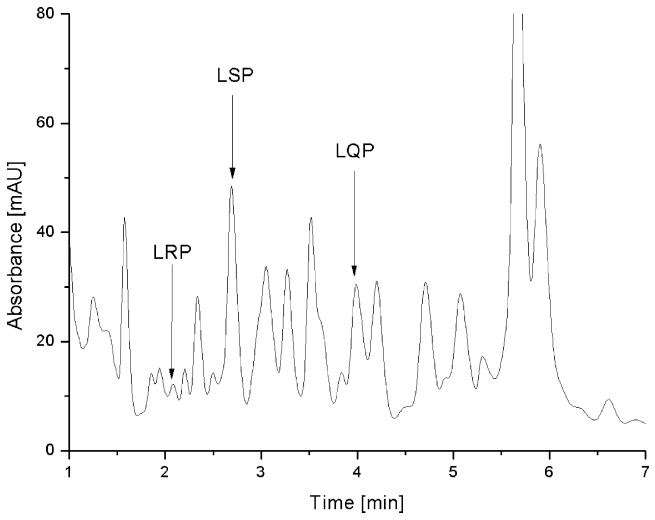

Fig. 4. Separation of LRP, LSP, and LQP peptides from the digested extracts corresponding to maize line B73 under the final chromatographic conditions: column: Ascentis Express Peptide ES-C18 (100 mm × 2.1 mm I.D., with 2.7 μm particle size) with Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 μm particle size), mobile phases: A, Milli-Q water/20 mM AA; B, ACN/20 mM AA; gradient: 3% B for 5 min, 3–5% B in 5 min, 5% B for 5 min, 5–97% B in 2 min, and 97–3% B in 2 min, T = 40 °C, inj. vol. = 5 μL, F = 0.4 mL/min, λ = 210 nm.

maize lines. Nevertheless, LRP is the most potential antihypertensive peptide (IC_{50} = 0.29 μM) causing a decrease in blood pressure of about 15 mmHg by intravenously injection of a small amount of peptide (30 mg/kg) in SHR (spontaneously hypertensive rats) after 2 min intake [35]. It should be highlighted that a reduction of the diastolic blood pressure of 5 mmHg decreases the risk of heart disease by approximately 16% [5]. In the case of peptides LQP (IC_{50} = 2.0 μM) and LSP (IC_{50} = 1.7 μM), their content significantly varied among the studied maize lines. The highest yield of LSP peptide was found in the EZ9 and EZ11A crops, while the smallest in the B73. The highest level of LQP was observed in the EZ11A line, and the lowest in the B73. Concluding the crop with the highest yield of antihypertensive peptides seemed to be the EZ11A line while the B73 crop showed the lowest content in antihypertensive peptides. The reproducibility of the method for each peptide and for every crop was calculated based on the area of two independently prepared samples injected twice into the HPLC. The reproducibility, expressed as RSD values, for each peptide was 7.28% for LRP, 1.43% for LSP, and 1.27% for LQP.

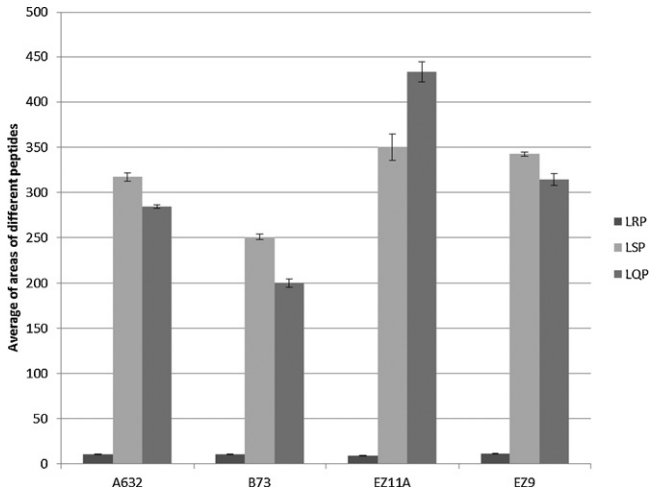

Fig. 5. Comparison of average areas of peptides LQP, LSP, and LRP in different maize varieties (four maize varieties, prepared twice and injected two times in the HPLC; 16 analysis).

4. Conclusions

According to the results presented in this work, it can be concluded that, a new methodology has been developed for extracting and purifying α-zeins from whole maize kernels. The method resulted precise, effective, and quick. A solvent has been selected enabling the suitable solubilization of precipitated α-zeins and their suitable digestion (after urea dilution to 6 M) with thermolysin. A method has been optimized for the digestion of α-zeins with thermolysin. Three antihypertensive peptides (LQP, LSP, and LRP) have been identified by HPLC–Q-TOF in the digests of extracted α-zeins from maize kernels. A RP-HPLC analytical methodology using a fused-core column was optimized enabling the separation of the three antihypertensive peptides in maize crops in less than 6 min after the optimized extraction and digestion of α-zeins from maize crops. The contents of LQP, LRP, and LSP peptides have been estimated in different maize varieties. The content of LRP peptide (IC_{50} = 0.29 μM) was very low regardless of the maize variety. LQP (IC_{50} = 2.0 μM) and LSP (IC_{50} = 1.7 μM) peptides, presenting an activity more than twice that of the most known and studied VPP and IPP peptides (IC_{50} = 9.13 and 5.15 μM, respectively), were detected in all maize varieties. Significant differences in the content of LQP and LSP were observed among studied maize lines which clearly demonstrated the different antihypertensive activity of maize lines.

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